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**PATENT**

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**In re Application of:**

Havenga et al.

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**For:** GENE DELIVERY VECTORS  
PROVIDED WITH A TISSUE TROPISM  
FOR DENDRITIC CELLS AND METHODS  
OF USE

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**CERTIFICATE OF MAILING**

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**COMMUNICATION**

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Sir:

Enclosed is a copy of Priority Document 98202297.2 EP filed July 8, 1998 for the above referenced application.

Respectfully submitted,

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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

**Patentanmeldung Nr.    Patent application No.    Demande de brevet n°**

98202297.2

Der Präsident des Europäischen Patentamts;  
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For the President of the European Patent Office

Le Président de l'Office européen des brevets  
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Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:  
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.  
If no title is shown please refer to the description.  
Si aucun titre n'est indiqué se référer à la description.)

Chimaeric adenoviruses

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Title: Chimaeric adenoviruses

The invention relates to the field of molecular genetics and medicine. In particular the present invention relates to the field of gene therapy, more in particular to gene therapy using viruses, especially adenoviruses.

5 In gene therapy, genetic information is delivered to a host cell in order to either correct (supplement) a genetic deficiency in said cell, or to inhibit an unwanted function in said cell, or to eliminate said host cell. Of course the genetic information can also be intended to provide the host  
10 cell with a wanted function, for instance to supply a secreted protein to treat other cells of the host, etc.

Thus there are basically three different approaches in gene therapy, one directed towards compensating a deficiency present in a (mammalian) host; the second directed towards  
15 the removal or elimination of unwanted substances (organisms or cells) and the third towards providing a cell with a wanted function.

For the purpose of gene therapy, adenoviruses have been  
20 proposed as suitable vehicles to deliver genes to the host. Gene-transfer vectors derived from adenoviruses (so-called adenoviral vectors) have a number of features that make them particularly useful for gene transfer. 1) the biology of the adenoviruses is characterized in detail, 2) the adenovirus  
25 is not associated with severe human pathology, 3) the virus is extremely efficient in introducing its DNA into the host cell, 4) the virus can infect a wide variety of cells and has a broad host-range, 5) the virus can be produced at high virus titers in large quantities, and 6) the virus can be  
30 rendered replication defective by deletion of the early-region 1 (E1) of the viral genome (Brody et al, 1994). However, there are still drawbacks associated with the use of adenoviral vectors. Typically adenoviruses, especially the well investigated serotypes usually elicit an immune  
35 response by a host into which they are introduced. Also,

although the virus generally spoken has a wide infection range, there is a problem in targeting certain cells and tissues. Also, the replication and other functions of the adenovirus are not always very well suited for the cells  
5 which are to be provided with the additional genetic material.

The adenovirus genome is a linear double-stranded DNA molecule of approximately 36000 base pairs. The adenovirus  
10 DNA contains identical Inverted Terminal Repeats (ITR) of approximately 90-140 base pairs with the exact length depending on the serotype. The viral origins of replication are within the ITRs exactly at the genome ends.  
Most adenoviral vectors currently used in gene therapy have  
15 a deletion in the E1 region, where novel genetic information can be introduced. The E1 deletion renders the recombinant virus replication defective (Levrero et al, 1991). It has been demonstrated extensively that recombinant adenovirus, in particular serotype 5 is suitable for efficient transfer  
20 of genes *in vivo* to the liver, the airway epithelium and solid tumors in animal models and human xenografts in immunodeficient mice (Bout, 1996; Blaese et al., 1995).  
Thus, preferred methods for *in vivo* gene transfer into target cells make use of adenoviral vectors as gene delivery  
25 vehicles.

At present, six different subgroups of human adenoviruses have been proposed which in total encompasses 51 distinct adenovirus serotypes (see table 1). Besides these human  
30 adenoviruses an extensive number of animal adenoviruses have been identified (see Ishibashi et al, 1983).

A serotype is defined on the basis of its immunological distinctiveness as determined by quantitative neutralization  
35 with animal antisera (horse, rabbit). If neutralization shows a certain degree of cross-reaction between two viruses,



distinctiveness of serotype is assumed if A) the hemagglutinins are unrelated, as shown by lack of cross-reaction on hemagglutination-inhibition, or B) substantial biophysical/ biochemical differences in DNA exist (Francki et al, 1991). The nine serotypes identified last (42-51) were isolated for the first time from HIV- infected patients (Hierholzer et al 1988; Schnurr et al 1993; De Jong et al 1998). For reasons not well understood, most of such immuno-compromised patients shed adenoviruses that were rarely or never isolated from immuno-competent individuals (Hierholzer et al 1988, 1992; Khoo et al, 1995, De Jong et al, 1998).

Besides differences towards the sensitivity against neutralising antibodies of different adenovirus serotypes, it has also been shown that adenoviruses in subgroup C such as Ad2, and Ad5 bind to different receptors as compared to adenoviruses from subgroup B such as Ad3 (Defer et al, 1990). Likewise, it was demonstrated that receptor specificity could be altered by exchanging the Ad3 with the Ad 5 knob protein, and vice versa (Krasnykh et al, 1996; Stevenson et al, 1995, 1997). The adenovirus serotype 5 is most widely used for gene therapy purposes. Similar to serotypes 2, 4 and 7, serotype 5 has a natural affiliation towards lung epithelia and other respiratory tissues. In contrast, it is known that, for instance, serotypes 40 and 41 have a natural affiliation towards the gastrointestinal tract. The serotypes described above, differ in at least capsid proteins (penton-base, hexon), proteins responsible for cell binding (fiber protein), and proteins involved in adenovirus replication.

One of the major problems of adenovirus gene therapy is thus that none of the above described serotypes are ideally suitable for delivering additional genetic material to host cells. Some have a somewhat limited host range, but have the benefit of being less immunogenic, some are the other way round. Some have a problem of being of a limited virulence,

but have a broad host range and/or a reduced immunogenicity. To make things even more complicated this variation in the adenovirus serotypes is also very dependent on the host to be treated. Some hosts may already have encountered certain serotypes and thus

mount a strong immune response to said serotype or a related serotype. Persons skilled in the art know that there are many other variations on this same theme.

The present invention now makes use of the fact that some adenoviruses have lower immunogenicity than others, which others typically excel in one of the other requirements for an efficient gene therapy regime, such as having a high specificity for a certain group of host cells, a good replication machinery in such host cells, a high rate of infection in certain host cells, etc. The invention thus provides chimaeric adenoviruses having the useful properties of at least two adenoviruses of different serotypes.

Typically, more than two requirements from the above non-exhaustive list are required to obtain an adenovirus capable of efficiently transferring additional material to a host cell and therefore the invention provides adenovirus derived vectors which can be used as cassettes to insert different adenoviral genes from different adenoviral serotypes at the required sites for obtaining a vector capable of expressing a chimaeric adenovirus, whereby of course also a gene of interest can be inserted at for instance the site of E1 of the original adenovirus from which the vector is derived. In this manner the chimaeric adenovirus to be produced can be adapted to the requirements and needs of certain hosts in need of gene therapy for certain disorders. Of course to enable this production a packaging cell will generally be needed in order to produce sufficient amount of safe chimaeric adenoviruses.

Thus in one embodiment the invention provides a chimaeric adenovirus comprising at least a part of a fiber protein and/or a protein involved in replication of an adenovirus

serotype providing the chimaeric virus with a desired host range and/or improved replication properties and at least a part of a penton or hexon protein from another less antigenic adenovirus serotype resulting in a less antigenic chimaeric adenovirus. Typically such a virus will be produced using a vector (typically a plasmid, a cosmid or baculovirus system which vector is of course also part of the present invention. A preferred vector is a vector which can be used to make a chimaeric recombinant virus specifically adapted to the host to be treated and the disorder to be treated. Such a vector is another embodiment of the present invention. Thus the invention also provides a recombinant vector derived from an adenovirus comprising at least one ITR and a packaging signal, having an insertion site for a nucleic acid sequence of interest, and further having an insertion site for functionally inserting a gene encoding a penton and/or a hexon protein of a first serotype of adenovirus and having an insertion site for a gene encoding a fiber protein of a second adenovirus of a different serotype, and/or an insertion site for a gene derived from a serotype having improved characteristics in the function carried out by that gene or its product. Typically the invention provides cassettes which allow for the production of any desired chimaeric adenovirus, be it only derived from two serotypes or as many as needed to obtain the desired characteristics, whereby it is not always necessary that all characteristics are the best when seen as single properties. It may not even be necessary, for instance, to always alter penton and/or hexon together with another part of adenovirus genes. Sometimes the immunogenicity needs not be altered together with other properties. However, it is preferred to use penton and/or hexon genes from less immunogenic adenovirus serotypes. An important feature of the present invention is the means to produce the chimaeric virus. Typically, one does not want an adenovirus batch to be administered to the host cell which contains replication competent adenovirus,

although this is not always true. In general therefor it is desired to omit a number of genes (but at least one) from the adenoviral genome on the vector encoding the chimaeric virus and to supply these genes in the genome of the cell in which the vector is brought to produce chimaeric adenovirus. Such a cell is usually called a packaging cell. The invention thus also provides a packaging cell for producing a chimaeric adenovirus according to the invention, comprising in trans all elements necessary for adenovirus production not present on the adenoviral vector according to the invention. Typically vector and packaging cell have to be adapted to one another in that they have all the necessary elements, but that they do not have overlapping elements which lead to replication competent virus by recombination. Thus the invention also provides a kit of parts comprising a packaging cell according to the invention and a recombinant vector according the invention whereby there is essentially no sequence overlap leading to recombination resulting in the production of replication competent adenovirus between said cell and said vector. In order to be able to precisely adapt the viral vector and provide the chimaeric virus with the desired properties at will, it is preferred that a library of adenoviral genes is provided whereby the genes are located within restriction sites. Typically it is preferred to have same kinds of genes of different serotypes within the same restriction sites and to have that same restriction site in the adenoviral vector used to produce the chimaeric virus. If all sites for different genes are unique then a system to pick and choose from has been made. One can cut a penton gene from the desired serotype from the library and insert it at the same site in the vector. One can then use a different restriction enzyme to cut a replication gene from the bank of a different serotype using another restriction enzyme and insert that gene at the corresponding restriction site in the chimaeric vector. Thus it is to be preferred to have a vector according

to the invention where the insertion sites are different and preferably unique restriction sites. Preferably this vector is combined with a library having the corresponding genes within the same restriction sites. Methods to use this  
5 library and the vector are within the skill in the art and are part of the present invention. Typically such a method comprises a number of restriction and ligation steps and expression of the result in a packaging cell. Also one can use a library from which the different desired adenoviral  
10 genes are obtained through homologous recombination or a combination of restriction and recombination. Thus the invention provides a method for producing a chimaeric adenovirus having a desired host range and deminished antigenicity, comprising providing a vector according to the  
15 invention having the desired insertion sites, inserting into said vector at least a functional part of a penton or hexon protein derived from an adenovirus serotype having relatively low antigenicity, inserting at least a functional part of a fiber protein derived from an adenovirus serotype having the  
20 desired host range and transfecting said vector in a packaging cell according to the invention and allowing for production of chimaeric viral particles. Of course other combinations of other viral genes originating from different serotypes can also be inserted as disclosed herein before.  
25 An immunogenic response to adenovirus that typically occurs is the production of neutralizing antibodies by the host. This is typically a reason for selecting a penton, hexon and/or fiber of a less immunogenic serotype. Of course it may not be necessary to make chimaeric  
30 adenoviruses which have complete proteins from different serotypes. It is well within the skill of the art to produce chimaeric proteins, for instance in the case of fiber proteins it is very well possible to have the base of one serotype and the shaft and the knob from another serotype.  
35 In this manner it becomes possible to have the parts of the protein responsible for assembly of viral particles

originate from one serotype, thereby enhancing the production of intact viral particles. Thus the invention also provides a chimaeric adenovirus according to the invention, wherein the hexon, penton and/or fiber proteins  
5 are chimaeric proteins originating from different adenovirus serotypes. Besides generating chimaeric adenoviruses by swapping entire wild type hexon, penton, fiber (protein) genes etc. or parts thereof, it is also within the scope of the present invention to insert hexon, penton, fiber  
10 (protein) genes etc. carrying mutations such as point mutations, deletions, insertions etc. which can be easily screened for preferred characteristics such as temperature stability, assembly, anchoring, redirected infection, altered immune response etc. Again other chimaeric  
15 combinations can also be produced and are within the scope of the present invention.

#### **Detailed description.**

20 It has been demonstrated in mice that upon *in vivo* systemic delivery of recombinant adenovirus serotype 5 for gene therapy purposes approximately 99% of the virus is trapped in the liver (Herz et al, 1993). Therefore, alteration of the adenovirus serotype 5 host cell range to be able to target  
25 other organs *in vivo* is a major interest of the invention, particularly in combination with other alterations, in particular the immunogenicity.

The initial step for successful infection is binding of  
30 adenovirus to its target cell, a process mediated through fiber protein. The fiber protein has a trimeric structure (Stouten et al, 1992), with different lengths depending on the virus serotype (Signas et al 1985; Kidd et al 1993). Different serotypes have polypeptides with structurally  
35 similar N and C termini, but different middle stem regions. N-terminally, the first 30 aminoacids are involved in

anchoring of the fiber to the penton base (Chroboczek et al, 1995), especially the conserved FNPVYP region in the tail (Arnberg et al 1997). The C-terminus, or knob, is responsible for initial interaction with the cellular

5 adenovirus receptor. After this initial binding secondary binding between the capsid penton base and cell-surface integrins leads to internalisation of viral particles in coated pits and endocytosis (Morgan et al, 1969; Svensson et al, 1984; Varga et al, 1992; Greber et al, 1993; Wickham et

10 al, 1994). Integrins are  $\alpha\beta$ -heterodimers of which at least 14  $\alpha$ -subunits and 8  $\beta$ -subunits have been identified (Hynes et al, 1992). The array of integrins expressed in cells is complex and will vary between cell types and cellular environment. Although the knob contains some conserved

15 regions, between serotypes, knob proteins show a high degree of variability, indicating that different adenovirus receptors exist. For instance, it has been demonstrated that adenoviruses of subgroup C (Ad2, Ad5) and adenoviruses of subgroup B (Ad3) bind to different receptors (Defner et al,

20 1990). The fiber protein also contains the type specific  $\gamma$ -antigen, which together with the  $\epsilon$ -antigen of the hexon determines the serotype specificity. The  $\gamma$ -antigen is localized on the fiber and it is known that it consists of 17 aminoacids (Eiz et al, 1997). The anti-fiber antibodies

25 of the host are therefore directed to the trimeric structure of the knob. The anti-fiber antibodies together with antibodies directed against the penton base and hexon proteins are responsible for the neutralisation of adenovirus particles. First the anti-fiber antibodies uncoat

30 the adenovirus particles after which the penton base is accessible to the anti-penton base antibodies (Gahery-Segard et al, 1998). Although this seems to be a very effective way to neutralize adenovirus particles others have described that the anti-hexon antibodies are the most effective ones

35 in neutralization of the particles (Gall et al, 1996).

To obtain re-directed infection of recombinant adenovirus serotype 5, several approaches have been or still are under investigation. Wickham et al has altered the RGD (Arg, Gly, Asp) motif in the penton base which is believed to be responsible for the  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrin binding to the penton base. They have replaced this RGD motif by another peptide motif which is specific for the  $\alpha_4\beta_1$  receptor. In this way targeting the adenovirus to a specific target cell could be accomplished (Wickham et al, 1995, 1996). Krasnykh et al has made use of the HI loop available in the knob. This loop is, based on X-ray crystallographics, located on the outside of the knob trimeric structure and therefore is thought not to contribute to the intramolecular interactions in the knob. Insertion of a FLAG coding sequence into the HI loop resulted in targeting of the adenovirus to target cells (Krasnykh et al, 1998). However, complete CAR independent infection was not observed.

It is an object of the present invention to provide a method and means by which adenoviruses can be constructed with an altered immune response, or with the absence or decreased infection in antigen presenting cells such as dendritic cells or macrophages. It is a further object of the present invention to provide methods for the generation of chimaeric adenoviruses as described above which can be targeted to specific cell types *in vitro* as well as *in vivo* have an altered tropism for certain cell types. It is a further object of the present invention to provide a method and means by which such an adenovirus can be used as a protein or nucleic acid delivery vehicle to a specific cell type or tissue.

The generation of chimaeric adenoviruses based on adenovirus serotype 5 with modified late genes is described. For this purpose, three plasmids, which together contain the complete



adenovirus serotype 5 genome, were constructed. From these plasmids the DNA encoding the adenovirus serotype 5 penton-base protein, hexon protein, and fiber protein were removed and replaced by linker DNA sequences which facilitate easy cloning. These plasmids subsequently served as template for the insertion of DNA encoding for penton-base protein, hexon protein, and fiber protein derived from different adenovirus, serotypes (human or animal). The DNAs derived from the different serotypes were obtained using the polymerase chain reaction technique in combination with (degenerate) oligonucleotides. At the former E1 location in the genome of adenovirus serotype 5, any gene of interest can be cloned. A single transfection procedure of the three plasmids together resulted in the formation of a recombinant chimaeric adenovirus. This new technology of libraries consisting of chimeric adenoviruses thus allows for a rapid screening for improved recombinant adenoviral vectors for *in vitro* and *in vivo* gene therapy purposes.

Although successful introduction of changes in the adenovirus serotype 5 fiber and penton-base have been reported, the complex structure of knob and the limited knowledge of the precise aminoacids interacting with CAR render such targeting approaches laborious and difficult. To overcome the limitations described above we used pre-existing adenovirus fibers, penton base proteins, and hexon proteins derived from other adenovirus serotypes. By generating chimeric adenovirus serotype 5 libraries containing structural proteins of alternative adenovirus serotypes, we have developed a technology which enables rapid screening for a recombinant adenoviral vector with preferred characteristics.

In one aspect this invention describes the use of chimeric adenoviruses to overcome, natural existing or induced,

neutralising host activity towards recombinant adenoviruses administered *in vivo* for therapeutic applications. The host immune response is predominantly directed against penton base - and hexon proteins present in the adenoviral capsid and to a lesser extent directed to fiber.

The adenovirus serotypes are defined by the inability to cross-react with neutralising antibodies in animal sera. Therefore chimaeric viruses based on for example adenovirus serotype 5 but chimeric for penton base protein, and/ or hexon protein provoke an altered, less severe immune response. The need for such chimaeric adenoviruses is stressed by observations that 1) repeated systemic delivery of recombinant adenovirus serotype 5 is unsuccessful due to formation of high titers of neutralising antibodies against the recombinant adenovirus serotype 5 (Schulick et al, 1997), and 2) pre-existing or natural immunity.

This aspect of the invention therefore circumvents the inability to repeat the administration of an adenovirus for gene therapy purposes. Preferably, the penton base-, hexon-, and fiber proteins are derived from adenoviruses in subgroup B and D and are more specifically of the adenovirus serotype 16, 24, 33, 36, 38, 39, 42, and 50. This latter is because these serotypes are rarely isolated from humans indicating that low titers of circulating neutralising antibodies are present against these serotypes.

In another aspect this invention describes chimaeric adenoviruses and methods to generate these viruses that have an altered tropism different from that of adenovirus serotype 5. For example, viruses based on adenovirus serotype 5 but displaying any adenovirus fiber existing in nature. This chimaeric adenovirus serotype 5 is able to infect certain cell types more efficiently, or less efficiently *in vitro* and *in vivo* than the adenovirus serotype 5. Such cells include but are not limited to endothelial cells, smooth muscle cells, dendritic cells,

neuronal cells, glial cells, synovial cells, lung epithelial cells, hemopoietic stem cells, monocytic/macrophage cells etc.

5 In another aspect this invention describes methods which identify chimeric adenoviruses that display improved in vitro amplification in static or suspension cell cultures. Adenoviruses derived from different subgroups, but also within one subgroup, display a high variability in  
10 productive infection in cell types that are used for production of recombinant adenovirus. Table 2 lists an overview of different adenovirus serotypes and their association with human disease, demonstrating that replication of a given adenovirus serotype is enhanced in  
15 certain cell types. For the production of recombinant adenoviruses for gene therapy purposes, several cell lines are available. These include but do not limit PER.C6, 911, 293, and E1 A549. These adenovirus producer cells may not be the most suited cell types to amplify adenovirus serotype 5  
20 based viruses. Therefore, in this aspect of the invention we select adenoviruses from different serotypes based on their ability to propagate for example on PER.C6 and use their early genes (without E1) and ITRs to construct chimeric viruses which are superior in terms of propagation and thus  
25 yield higher titers as compared to commonly used adenovirus serotype 2 or 5.

In another aspect the invention describes the construction and use of libraries consisting of distinct parts of  
30 adenovirus serotype 5 in which one or more genes or sequences have been replaced with DNA derived from alternative human or animal serotypes. This set of constructs, in total encompassing the complete adenovirus genome, allows for the construction of unique chimeric  
35 adenoviruses customised for a certain group of patients or even a single individual.

In all aspects of the invention the chimeric adenoviruses may, or may not, contain deletions in the E1 region and insertions of heterologous genes linked either or not to a promoter. Furthermore, chimeric adenoviruses may, or may not, contain deletions in the E3 region and insertions of heterologous genes linked to a promoter. Furthermore, chimeric adenoviruses may, or may not, contain deletions in the E2 and/ or E4 region and insertions of heterologous genes linked to a promoter. In the latter case E2 and/ or E4 complementing cell lines are required to generate recombinant adenoviruses.

**Example 1: Generation of adenovirus serotype 5 genomic plasmid clones**

The complete genome of adenovirus serotype 5 has been cloned into various plasmids or cosmids to allow easy modification of parts of the adenovirus serotype 5 genome, while still retaining the capability to produce recombinant virus. For this purpose the following plasmids were generated:

1. pBr/Ad.Bam-rITR (ECACC deposit P97082122)

In order to facilitate blunt end cloning of the ITR sequences, wild-type human adenovirus type 5 (Ad5) DNA was treated with Klenow enzyme in the presence of excess dNTPs. After inactivation of the Klenow enzyme and purification by phenol/chloroform extraction followed by ethanol precipitation, the DNA was digested with BamHI. This DNA preparation was used without further purification in a ligation reaction with pBr322 derived vector DNA prepared as follows: pBr322 DNA was digested with EcoRV and BamHI, dephosphorylated by treatment with TSAP enzyme (Life Technologies) and purified on LMP agarose gel (SeaPlaque GTG). After transformation into competent *E.coli* DH5a (Life Techn.) and analysis of ampiciline resistant colonies, one

clone was selected that showed a digestion pattern as expected for an insert extending from the BamHI site in Ad5 to the right ITR.

Sequence analysis of the cloning border at the right ITR

5 revealed that the most 3' G residue of the ITR was missing, the remainder of the ITR was found to be correct. Said missing G residue is complemented by the other ITR during replication.

10 2. pBr/Ad.Sal-rITR (ECACC deposit P97082119)

pBr/Ad.Bam-rITR was digested with BamHI and SalI. The vector fragment including the adenovirus insert was isolated in LMP agarose (SeaPlaque GTG) and ligated to a 4.8 kb SalI-BamHI fragment obtained from wt Ad5 DNA and purified with the  
15 Geneclean II kit (Bio 101, Inc.). One clone was chosen and the integrity of the Ad5 sequences was determined by restriction enzyme analysis. Clone pBr/Ad.Sal-rITR contains adeno type 5 sequences from the SalI site at bp 16746 up to and including the rITR (missing the most 3' G residue).

20

3. pBr/Ad.Cla-Bam (ECACC deposit P97082117)

wt Adeno type 5 DNA was digested with ClaI and BamHI, and the 20.6 kb fragment was isolated from gel by electro-elution. pBr322 was digested with the same enzymes and purified from  
25 agarose gel by Geneclean. Both fragments were ligated and transformed into competent DH5a. The resulting clone pBr/Ad.Cla-Bam was analysed by restriction enzyme digestion and shown to contain an insert with adenovirus sequences from bp 919 to 21566.

30

4. pBr/Ad.AflII-Bam (ECACC deposit P97082114)

Clone pBr/Ad.Cla-Bam was linearized with EcoRI (in pBr322) and partially digested with AflII. After heat inactivation of AflII for 20' at 65°C the fragment ends were filled in with  
35 Klenow enzyme. The DNA was then ligated to a blunt double stranded oligo linker containing a PacI site (5'-

AATTGTCTTAATTAACCGCTTAA-3'). This linker was made by annealing the following two oligonucleotides: 5'-AATTGTCTTAATTAACCGC-3' and 5'-AATTGCGGTTAATTAAGAC-3', followed by blunting with Klenow enzyme. After precipitation of the ligated DNA to change buffer, the ligations were digested with an excess PacI enzyme to remove concatameres of the oligo. The 22016 bp partial fragment containing Ad5 sequences from bp 3534 up to 21566 and the vector sequences, was isolated in LMP agarose (SeaPlaque GTG), religated and transformed into competent DH5a. One clone that was found to contain the PacI site and that had retained the large adeno fragment was selected and sequenced at the 5' end to verify correct insertion of the PacI linker in the (lost) AflIII site.

15

5. pBr/Ad.Bam-rITRpac#2 (ECACC deposit P97082120) and pBr/Ad.Bam-rITR#8 (ECACC deposit P97082121)

To allow insertion of a PacI site near the ITR of Ad5 in clone pBr/Ad.Bam-rITR about 190 nucleotides were removed between the ClaI site in the pBr322 backbone and the start of the ITR sequences. This was done as follows: pBr/Ad.Bam-rITR was digested with ClaI and treated with nuclease Bal31 for varying lengths of time (2', 5', 10' and 15'). The extent of nucleotide removal was followed by separate reactions on pBr322 DNA (also digested at the ClaI site), using identical buffers and conditions. Bal31 enzyme was inactivated by incubation at 75°C for 10 minutes, the DNA was precipitated and resuspended in a smaller volume of TE buffer. To ensure blunt ends, DNAs were further treated with T4 DNA polymerase in the presence of excess dNTPs. After digestion of the (control) pBr322 DNA with SalI, satisfactory degradation (~150 bp) was observed in the samples treated for 10' or 15'. The 10' or 15' treated pBr/Ad.Bam-rITR samples were then ligated to the above described blunted PacI linkers (See pBr/Ad.AflIII-Bam). Ligations were purified by precipitation, digested with excess PacI and separated from the linkers on

an LMP agarose gel. After religation, DNAs were transformed into competent DH5a and colonies analyzed. Ten clones were selected that showed a deletion of approximately the desired length and these were further analyzed by T-track sequencing (T7 sequencing kit, Pharmacia Biotech). Two clones were found with the PacI linker inserted just downstream of the rITR. After digestion with PacI, clone #2 has 28 bp and clone #8 has 27 bp attached to the ITR.

10 pWE/Ad.AflIII-rITR (ECACC deposit P97082116)

Cosmid vector pWE15 (Clontech) was used to clone larger Ad5 inserts. First, a linker containing a unique PacI site was inserted in the EcoRI sites of pWE15 creating pWE.pac. To this end, the double stranded PacI oligo as described for pBr/Ad.AflIII-BamHI was used but now with its EcoRI protruding ends. The following fragments were then isolated by electro-elution from agarose gel: pWE.pac digested with PacI, pBr/AflIII-Bam digested with PacI and BamHI and pBr/Ad.Bam-rITR#2 digested with BamHI and PacI. These fragments were ligated together and packaged using 1 phage packaging extracts (Stratagene) according to the manufacturer's protocol. After infection into host bacteria, colonies were grown on plates and analyzed for presence of the complete insert. pWE/Ad.AflIII-rITR contains all adenovirus type 5 sequences from bp 3534 (AflIII site) up to and including the right ITR (missing the most 3' G residue).

pBr/Ad.lITR-Sal(9.4) (ECACC deposit P97082115)

Adeno 5 wt DNA was treated with Klenow enzyme in the presence of excess dNTPs and subsequently digested with SalI. Two of the resulting fragments, designated left ITR-Sal(9.4) and Sal(16.7)-right ITR, respectively, were isolated in LMP agarose (Seaplaque GTG). pBr322 DNA was digested with EcoRV and SalI and treated with phosphatase (Life Technologies). The vector fragment was isolated using the GeneClean method (BIO 101, Inc.) and ligated to the Ad5 SalI fragments. Only

the ligation with the 9.4 kb fragment gave colonies with an insert. After analysis and sequencing of the cloning border a clone was chosen that contained the full ITR sequence and extended to the SalI site at bp 9462.

5

pBr/Ad.lITR-Sal(16.7) (ECACC deposit P97082118)

pBr/Ad.lITR-Sal(9.4) is digested with SalI and dephosphorylated (TSAP, Life Technologies). To extend this clone upto the third SalI site in Ad5, pBr/Ad.Cla-Bam was linearized with BamHI and partially digested with SalI. A 7.3 kb SalI fragment containing adenovirus sequences from 9462-16746 was isolated in LMP agarose gel and ligated to the SalI-digested pBr/Ad.lITR-Sal(9.4) vector fragment.

15 pWE/Ad.AflIII-EcoRI

pWE.pac was digested with ClaI and 5' protruding ends were filled using Klenow enzyme. The DNA was then digested with PacI and isolated from agarose gel. pWE/AflIII-rITR was digested with EcoRI and after treatment with Klenow enzyme digested with PacI. The large 24 kb fragment containing the adenoviral sequences was isolated from agarose gel and ligated to the ClaI-digested and blunted pWE.pac vector using the Ligation Express<sup>tm</sup> kit from Clontech. After transformation of Ultracompetent XL10-Gold cells from Stratagene, clones were identified that contained the expected insert. pWE/AflIII-EcoRI contains Ad5 sequences from bp 3534-27336.

#### Construction of new adapter plasmids

30 The absence of sequence overlap between the recombinant adenovirus and E1 sequences in the packaging cell line is essential for safe, RCA-free generation and propagation of new recombinant viruses. The adapter plasmid pMLPI.TK (figure. 1) is an example of an adapter plasmid designed for use according to the invention in combination with the improved packaging cell lines of the invention. This plasmid

35



was used as the starting material to make a new vector in which nucleic acid molecules comprising specific promoter and gene sequences can be easily exchanged.

First, a PCR fragment was generated from pZip $\Delta$ Mo+PyF101(N<sup>-</sup>)  
 5 template DNA (described in PCT/NL96/00195) with the following  
 primers: LTR-1: 5'-CTG TAC GTA CCA GTG CAC TGG CCT AGG CAT  
 GGA AAA ATA CAT AAC TG-3' and LTR-2: 5'-GCG GAT CCT TCG AAC  
 CAT GGT AAG CTT GGT ACC GCT AGC GTT AAC CGG GCG ACT CAG TCA  
 ATC G-3'. Pwo DNA polymerase (Boehringer Mannheim) was used  
 10 according to manufacturers protocol with the following  
 temperature cycles: once 5' at 95°C; 3' at 55°C; and 1' at  
 72°C, and 30 cycles of 1' at 95°C, 1' at 60°C, 1' at 72°C,  
 followed by once 10' at 72°C. The PCR product was then  
 digested with BamHI and ligated into pMLP10 (Levrero *et al.*,  
 15 1991) vector digested with PvuII and BamHI, thereby  
 generating vector pLTR10. This vector contains adenoviral  
 sequences from bp 1 up to bp 454 followed by a promoter  
 consisting of a part of the Mo-MuLV LTR having its wild-type  
 enhancer sequences replaced by the enhancer from a mutant  
 20 polyoma virus (PyF101). The promoter fragment was designated  
 L420. Next, the coding region of the murine HSA gene was  
 inserted. pLTR10 was digested with BstBI followed by Klenow  
 treatment and digestion with NcoI. The HSA gene was obtained  
 by PCR amplification on pUC18-HSA (Kay *et al.*, 1990) using  
 25 the following primers: HSA1, 5'-GCG CCA CCA TGG GCA GAG CGA  
 TGG TGG C-3' and HSA2, 5'-GTT AGA TCT AAG CTT GTC GAC ATC GAT  
 CTA CTA ACA GTA GAG ATG TAG AA-3'. The 269 bp amplified  
 fragment was subcloned in a shuttle vector using the NcoI and  
 BglII sites. Sequencing confirmed incorporation of the  
 30 correct coding sequence of the HSA gene, but with an extra  
 TAG insertion directly following the TAG stop codon. The  
 coding region of the HSA gene, including the TAG duplication  
 was then excised as a NcoI (sticky)-SalI (blunt) fragment and  
 cloned into the 3.5 kb NcoI(sticky)/BstBI(blunt) fragment  
 35 from pLTR10, resulting in pLTR-HSA10.

Finally, pLTR-HSA10 was digested with EcoRI and BamHI after which the fragment containing the left ITR, packaging signal, L420 promoter and HSA gene was inserted into vector pMLPI.TK digested with the same enzymes and thereby replacing the  
 5 promoter and gene sequences. This resulted in the new adapter plasmid pAd/L420-HSA (figure. 2) that contains convenient recognition sites for various restriction enzymes around the promoter and gene sequences. SnaBI and AvrII can be combined with HpaI, NheI, KpnI, HindIII to exchange promoter  
 10 sequences, while the latter sites can be combined with the ClaI or BamHI sites 3' from HSA coding region to replace genes in this construct.

Another adapter plasmid that was designed to allow easy exchange of nucleic acid molecules was made by replacing the  
 15 promoter, gene and poly A sequences in pAd/L420-HSA with the CMV promoter, a multiple cloning site, an intron and a poly-A signal. For this purpose, pAd/L420-HSA was digested with AvrII and BglII followed by treatment with Klenow to obtain blunt ends. The 5.1 kb fragment with pBr322 vector and  
 20 adenoviral sequences was isolated and ligated to a blunt 1570 bp fragment from pcDNA1/amp (Invitrogen) obtained by digestion with HhaI and AvrII followed by treatment with T4 DNA polymerase. This adapter plasmid was named pCLIP (figure. 3).

25

#### Generation of recombinant adenoviruses

- To generate E1 deleted recombinant adenoviruses with the new plasmid-based system, the following constructs are prepared:
- 30 a) An adapter construct containing the expression cassette with the gene of interest linearised with a restriction enzyme that cuts at the 3' side of the overlapping adenoviral genome fragment, preferably not containing any pBr322 vector sequences, and
  - 35 b) A complementing adenoviral genome construct pWE/Ad.AflIII-rITR digested with PacI.

These two DNA molecules are further purified by phenol/  
chloroform extraction and EtOH precipitation. Co-transfection  
of these plasmids into an adenovirus packaging cell line,  
preferably a cell line according to the invention, generates  
5 recombinant replication deficient adenoviruses by a one-step  
homologous recombination between the adapter and the  
complementing construct (figure. 4).

Alternatively, in stead of pWE/Ad.AflIII-rITR other fragments  
can be used, e.g., pBr/Ad.Cla-Bam digested with EcoRI and  
10 BamHI or pBr/Ad.AflIII-BamHI digested with PacI and BamHI can  
be combined with pBr/Ad.Sal-rITR digested with SalI. In this  
case, three plasmids are combined and two homologous  
recombinations are needed to obtain a recombinant adenovirus  
(figure. 5). It is to be understood that those skilled in the  
15 art may use other combinations of adapter and complementing  
plasmids without departing from the present invention.

A general protocol as outlined below and meant as a non-  
limiting example of the present invention has been performed  
to produce several recombinant adenoviruses using various  
20 adapter plasmids and the Ad.AflIII-rITR fragment. Adenovirus  
packaging cells (PER.C6) were seeded in  $\sim 25 \text{ cm}^2$  flasks and  
the next day when they were at  $\sim 80\%$  confluency, transfected  
with a mixture of DNA and lipofectamine agent (Life Techn.)  
as described by the manufacturer. Routinely, 40  $\mu\text{l}$   
25 lipofectamine, 4  $\mu\text{g}$  adapter plasmid and 4  $\mu\text{g}$  of the  
complementing adenovirus genome fragment AflIII- rITR (or 2  $\mu\text{g}$   
of all three plasmids for the double homologous  
recombination) are used. Under these conditions transient  
transfection efficiencies of  $\sim 50\%$  (48 hrs post transfection)  
30 are obtained as determined with control transfections using a  
pAd/CMV-LacZ adapter. Two days later, cells are passaged to  
 $\sim 80 \text{ cm}^2$  flasks and further cultured. Approximately five (for  
the single homologous recombination) to eleven days (for the  
double homologous recombination) later a cytopathogenic  
35 effect (CPE) is seen, indicating that functional adenovirus  
has formed. Cells and medium are harvested upon full CPE and

recombinant virus is released by freeze-thawing. An extra amplification step in an 80 cm<sup>2</sup> flask is routinely performed to increase the yield since at the initial stage the titers are found to be variable despite the occurrence of full CPE.

5 After amplification, viruses are harvested and plaque purified on PER.C6 cells. Individual plaques are tested for viruses with active transgenes.

Besides replacements in the E1 region it is possible to

10 delete or replace (part of) the E3 region in the adenovirus because E3 functions are not necessary for the replication, packaging and infection of the (recombinant) virus. This creates the opportunity to use a larger insert or to insert more than one gene without exceeding the maximum package size

15 (approximately 105% of wt genome length). This can be done, e.g., by deleting part of the E3 region in the pBr/Ad.Bam-rITR clone by digestion with XbaI and religation. This removes Ad5 wt sequences 28592-30470 including all known E3 coding regions. Another example is the precise replacement of

20 the coding region of gp19K in the E3 region with a polylinker allowing insertion of new sequences. This, 1) leaves all other coding regions intact and 2) obviates the need for a heterologous promoter since the transgene is driven by the E3 promoter and pA sequences, leaving more space for coding

25 sequences.

To this end, the 2.7 kb EcoRI fragment from wt Ad5 containing the 5' part of the E3 region was cloned into the EcoRI site of pBluescript (KS<sup>-</sup>) (Stratagene). Next, the HindIII site in the polylinker was removed by digestion with EcoRV and HincII

30 and subsequent religation. The resulting clone pBS.Eco-Eco/ad5DHIII was used to delete the gp19K coding region. Primers 1 (5'-GGG TAT TAG GCC AA AGG CGC A-3') and 2 (5'-GAT CCC ATG GAA GCT TGG GTG GCG ACC CCA GCG-3') were used to amplify a sequence from pBS.Eco-Eco/Ad5DHIII corresponding to

35 sequences 28511 to 28734 in wt Ad5 DNA. Primers 3 (5'-GAT CCC ATG GGG ATC CTT TAC TAA GTT ACA AAG CTA-3') and 4 (5'-GTC GCT

GTA GTT GGA CTG G-3') were used on the same DNA to amplify Ad5 sequences from 29217 to 29476. The two resulting PCR fragments were ligated together by virtue of the new introduced NcoI site and subsequently digested with XbaI and MunI. This fragment was then ligated into the pBS.Eco-Eco/ad5ΔHIII vector that was digested with XbaI (partially) and MunI generating pBS.Eco-Eco/ad5ΔHIII.Δgp19K. To allow insertion of foreign genes into the HindIII and BamHI site, an XbaI deletion was made in pBS.Eco-Eco/ad5ΔHIII.Δgp19K to remove the BamHI site in the Bluescript polylinker. The resulting plasmid pBS.Eco-Eco/ad5ΔHIIIΔgp19KΔXbaI, contains unique HindIII and BamHI sites corresponding to sequences 28733 (HindIII) and 29218 (BamHI) in Ad5. After introduction of a foreign gene into these sites, either the deleted XbaI fragment is re-introduced, or the insert is recloned into pBS.Eco-Eco/ad5ΔHIII.Δgp19K using HindIII and for example MunI. Using this procedure, we have generated plasmids expressing HSV-TK, hIL-1α, rat IL-3, luciferase or LacZ. The unique SrfI and NotI sites in the pBS.Eco-Eco/ad5ΔHIII.Δgp19K plasmid (with or without inserted gene of interest) are used to transfer the region comprising the gene of interest into the corresponding region of pBr/Ad.Bam-rITR, yielding construct pBr/Ad.Bam-rITRΔgp19K (with or without inserted gene of interest). This construct is used as described *supra* to produce recombinant adenoviruses. In the viral context, expression of inserted genes is driven by the adenovirus E3 promoter.

Recombinant viruses that are both E1 and E3 deleted are generated by a double homologous recombination procedure as described above for E1-replacement vectors using a plasmid-based system consisting of:

- a) an adapter plasmid for E1 replacement according to the invention, with or without insertion of a first gene of interest,
- b) the pWE/Ad.AflIII-EcoRI fragment, and

c) the pBr/Ad.Bam-rITR $\Delta$ gp19K plasmid with or without insertion of a second gene of interest.

In addition to manipulations in the E3 region, changes of (parts of) the E4 region can be accomplished easily in pBr/Ad.Bam-rITR. Generation and propagation of such a virus, however, in some cases demands complementation *in trans*.

## Example 2: Generation of adenovirus serotype 5 based viruses with chimeric fiber proteins

The method described *infra* to generate recombinant adenoviruses by co-transfection of two, or more separate cloned adenovirus sequences. These cloned adenoviral sequences were subsequently used to remove specific adenovirus serotype 5 sequences in order to generate "template clones" which allow for the easy introduction of DNA sequences derived from other adenovirus serotypes. As an example of these template clones, the construction of plasmids enabling swapping of DNA encoding for fiber protein is given.

### Generation of adenovirus template clones lacking DNA encoding for fiber

Fiber coding sequence of adenovirus serotype 5 is located between nucleotides 31042 and 32787. To remove the adenovirus serotype 5 DNA encoding fiber we started with construct pBr/Ad.Bam-rITR. From this construct first a NdeI site was removed. For this purpose, a pBr plasmid was first digested with NdeI after which protruding ends were filled using klenow. This pBr plasmid was then re-ligated, digested with NdeI after which the DNA was transformed into *E.coli* DH5 $\alpha$ . Since linear DNA can not enter *E.coli*, ampicillin resistant colonies obtained contain the desired pBr/ $\Delta$ NdeI plasmid. In the ScaI-SalI digested pBr / $\Delta$ NdeI plasmid we subsequently cloned the ScaI-SalI fragment derived from

pBr/Ad.BamrITR, resulting in plasmid pBr/Ad.Bam-rITRΔNdeI which hence contained a unique NdeI site. Next a PCR was performed with oligonucleotides ``NY-up'' and ``NY-down'' (figure 6). During amplification, both a NdeI and a NsiI  
 5 restriction site were introduced to facilitate cloning of the amplified fiber DNAs. Amplification consisted of 25 cycles of each 45 sec. at 94°C, 1 min. at 60°C, and 45 sec. at 72°C. The PCR reaction contained 25 pmol of oligonucleotides NY-up or NY-down, 2mM dNTP, PCR buffer with 1.5 mM MgCl<sub>2</sub>, and 1  
 10 unit of elongase heat stable polymerase (Gibco, the Netherlands). One-tenth of the PCR product was run on an agarose gel which demonstrated that the expected DNA fragment of ± 2200 bp was amplified. This PCR fragment was subsequently purified using Geneclean kit system. (Bio101 Inc)  
 15 Then, both the construct pBr/Ad.Bam-rITRΔNdeI as well as the PCR product were digested with restriction enzymes NdeI and SbfI. The PCR fragment was subsequently cloned using T4 ligase enzyme into the NdeI and SbfI sites thus generating pBr/Ad.BamRAFib.  
 20 Next, was the amplification of DNA sequences encoding for fiber protein derived from alternative adenovirus serotype DNAs.

#### Amplification of fiber sequences from adenovirus serotypes

25 To enable amplification of the DNAs encoding fiber protein derived from alternative serotypes degenerate oligonucleotides were synthesized. For this purpose, first known DNA sequences encoding for fiber protein of alternative serotypes were aligned to identify conserved regions in both  
 30 the tail-region as well as the knob-region of the fiber protein. From the alignment, which contained the nucleotide sequence of 19 different serotypes representing all 6 subgroups, (degenerate) oligonucleotides were synthesised (see table 3). Also shown in table 3 is the combination of  
 35 oligonucleotides used to amplify the DNA encoding fiber protein of a specific serotype. The amplification reaction

(50 µl) contained 2 mM dNTPs, 25 pmol of each oligonucleotide, standard 1x PCR buffer, 1,5 mM MgCl<sub>2</sub>, and 1 Unit Pwo heat stable polymerase (Boehringer) per reaction. The cycler program contained 20 cycles, each consisting of 30 sec. 94°C, 60 sec. 60-64°C, and 120 sec. At 72°C. One-tenth of the PCR product was run on an agarose gel which demonstrated that a DNA fragment was amplified. Of each different template, two independent PCR reactions were performed after which the independent PCR fragments obtained were sequenced to determine the nucleotide sequence. From 11 different serotypes, the nucleotide sequence could be compared to sequences present in the genbank. Of six other serotypes, i.e. 28, 32, 36, 49, 50, and 51, the DNA encoding fiber protein was previously unknown.

15

#### Generation of chimaeric adenoviral DNA constructs

All amplified fiber DNAs as well as the vector (pBr/Ad.BamRAFib) were digested with NdeI and NsiI. The digested DNAs was subsequently run on a agarose gel after which the fragments were isolated from the gel and purified using the Geneclean kit (Bio101 Inc). The PCR fragments were then cloned into the NdeI and NsiI sites of pBr/AdBamRAFib, thus generating pBr/AdBamRFibXX (where XX stands for the serotype number of which the fiber DNA was isolated). So far constructs of serotypes 5/8/9/12/16/19/28/32/36/37/40/41/49/50/51 have been produced.

25

#### Generation of recombinant adenovirus chimeric for fiber protein

Three constructs, pCLIP, pWE/AdAflIII-Eco and pBr/AdBamrITR.pac/fibXX were transfected into adenovirus producer cells. For transfection, 2 µg of pCLIP, and 4 µg of both pWE/AdAflIII-Eco and pBr/AdBamrITR.pac/fibXX were diluted in serum free DMEM to 100 µl total volume. To this DNA suspension 100 µl 1x diluted lipofectamine (Gibco) was added. After 30 minutes at room temperature the DNA-lipofectamine

30

35



complex solution was added to 2.5 ml of serum-free DMEM which was subsequently added to a T25 cm<sup>2</sup> tissue culture flask. This flask contained  $2 \times 10^6$  PER.C6 cells that were seeded 24-hours prior to transfection. Two hours later, the DNA-lipofectamine complex containing medium was diluted once by the addition of 2.5 ml DMEM supplemented with 20% fetal calf serum. Again 24 hours later the medium was replaced by fresh DMEM supplemented with 10% fetal calf serum. Cells were cultured for 6-8 days, subsequently harvested, and freeze/thawed 3 times. Cellular debris was removed by centrifugation for 5 minutes at 3000 rpm room temperature. Of the supernatant (12.5 ml) 3-5 ml was used to infect again PER.C6 cells (T80 cm<sup>2</sup> tissue culture flasks). This re-infection results in full cytopathogenic effect (CPE) after 5-6 days after which the adenovirus is harvested as described above. With the generated virus batch two assays were routinely performed. 1) 20  $\mu$ l virus supernatant, diluted 10-fold by the addition of 1980  $\mu$ l DMEM was used to infect human cell line A549 that were seeded 24-hours prior to infection at a concentration of  $10^5$  cells per well of 6-well plates. Forty-eight hours later protein lysates were prepared that were subsequently used to measure marker gene expression (luciferase activity). 2) 20  $\mu$ l virus supernatant is used to determine the virus titer on human 911 cells. For this purpose, 911 cells are seeded at a concentration of  $4 \times 10^4$  cells per well in 96-well plates. Three to four hours after seeding, the medium was replaced by adenovirus supernatant (dilution range: 2  $\mu$ l -  $5 \times 10^{-9}$   $\mu$ l). The virus titers of the chimeric fiber adenovirus serotype 5 always exceeded  $1 \times 10^8$  infectious units per ml.

#### Re-directed infection of chimeric adenoviruses

To demonstrate re-directed infection *in vitro* of the adenoviruses chimaeric for fiber protein, a panel of human

cell lines of different origins were used. This panel includes human hepatic cells, dendritic cells, hemopoietic cells, and smooth muscle cells. In these experiments wildtype adenovirus serotypes as well as recombinant adenovirus serotype 5 are included.

Target cells are seeded at a concentration of  $10^5$  cells per well of 6-well plates. Twenty-four hours later the medium is replaced by fresh medium containing the chimaeric adenoviruses at an MOI of approximately 10. Forty-eight hours later cells are harvested, washed and pelleted by centrifugating 5 minutes at 1500 rpm. Cells are subsequently lysed in 0,1 ml lysisbuffer (1% Triton-X-100, 10% Glycerol, 2 mM EDTA, 2 mM DTT, and 25 mM Tris-phosphate pH 7.8) after which the total protein concentration is measured (Biorad protein standard II). A volume corresponding to twenty micrograms of protein is subsequently used to determine marker gene expression (luciferase activity). To enable tracking of infection of the wildtype adenovirus serotypes, these viruses are labeled with radioactive  $^{125}\text{I}$  or fluorescence probes prior to infection. Using fluorescent microscopy or a measuring radioactivity, the efficiency of infection of different serotypes into particular cell types is determined.

To demonstrate re-directed infection *in vivo* of adenovirus chimeric for fiber protein  $1 \times 10^9$  infectious particles were injected via the tail vein into CBA/ ca mice (2 mice for each chimeric adenovirus). Detection of adenovirus infection into specific tissues is monitored on two different levels: 1) Binding of chimeric adenovirus is monitored by radioactive labelling the adenovirus (Eisenlohr et al, 1987; Matlin et al, 1981; Richman et al, 1986) or with fluorescent probes (Leopold et al, 1998). One hour after *in vivo* systemic delivery via the tail vein mice are sacrificed after which preferred binding is investigated by measuring radioactivity

in various organs c.q. tissues. 2) Successful infection is monitored by adenovirus gene expression of the marker gene i.e. LacZ or luciferase activity. Four days after administration mice are sacrificed after which organs and tissues are isolated. Samples included liver, spleen, gastrointestinal tract, peripheral blood, bone marrow, aorta, muscle, etc. Using this strategy, preferred binding of chimeric adenovirus towards tissues of interest can be investigated. Moreover, using this strategy, preferred infection of chimeric adenovirus into specific cells of particular organs can be determined.

**Example 3: Generation of adenovirus serotype 5 based viruses with chimeric hexon protein.**

The method described *infra* to generate recombinant adenoviruses by co-transfection of two, or more separate cloned adenovirus sequences. These cloned adenoviral sequences were subsequently used to remove specific adenovirus serotype 5 sequences in order to generate "template clones" which allow for the easy introduction of DNA sequences derived from other adenovirus serotypes. As an example of these template clones, the construction of plasmids enabling swapping of DNA encoding for hexon protein is given.

25

Generation of adenovirus template clones lacking DNA encoding for hexon

Hexon coding sequences of adenovirus serotype 5 are located between nucleotides 18841 and 21697. Since hexon sequences are still present at the extreme 3'-end of both constructs used for homologous recombination in adenovirus packaging cells to generate virus, we first removed these hexon sequences. For this purpose, construct pBr/Ad.BamrITR.pac#2 was digested with restriction enzyme NruI and subsequently religated, resulting in construct pBr/Ad.Nru-rITR. Restriction enzyme NruI is located 300 bp downstream of the

BamHI site and 600 bp upstream of the BamHI site, hence the 300 bp hexon sequence at the 3'-end of pBr/Ad.BamrITR was removed.

5 To facilitate easy cloning of hexon coding sequences from alternative adenovirus serotypes into the adenovirus serotype 5 backbone, first a subclone was generated. This subclone, coded pN/Ad.AscI, was generated by first digesting plasmid pNEB with BamHI and SbfI. Fill in of protruding ends and  
10 religation resulted in plasmid pNEB/ $\Delta$ Bam/Sbf. Plasmid pNEB/ $\Delta$ Bam/Sbf served as vector for the insertion of the adenovirus AscI fragment derived from pWE/Ad.AflIII-Eco. Deletion in pN/Ad.AscI of the adenovirus serotype 5 DNA encoding hexon was accomplished by generation of two PCR  
15 fragments. For these PCR reactions four different oligonucleotides were required:  $\Delta$ hex1- $\Delta$ hex4 (figure 7). The amplified DNA product of  $\pm$  1100 bp obtained with oligonucleotides  $\Delta$ hex1 and  $\Delta$ hex2 was digested with BamHI and FseI. The amplified DNA product of  $\pm$  1600 bp obtained with  
20 oligonucleotides  $\Delta$ hex3 and  $\Delta$ hex4 was digested with BamHI and SbfI. These digested PCR fragments were subsequently purified from agarose gel and in a tri-part ligation reaction using T4 ligase enzyme linked to pN/Ad.AscI digested with FseI and SbfI. The resulting construct was coded pN/Ad.AscI $\Delta$ Hex. This  
25 construct was sequenced in part to confirm the correct nucleotide sequence and the presence of unique restriction sites ClaI and AflIII.

#### Amplification of hexon sequences from adenovirus serotypes

30 To enable amplification of the DNAs encoding hexon protein derived from alternative serotypes degenerate oligonucleotides were synthesized. For this purpose, first known DNA sequences encoding for hexon protein of alternative serotypes were aligned to identify conserved regions in both  
35 the tail-region as well as the knob-region of the fiber protein. From the alignment, which contained the nucleotide

sequence of 9 different serotypes representing 5 of the 6 known subgroups, (degenerate) oligonucleotides were synthesised. These oligonucleotides were coded HEX-up (5'- GG ACGTGT AAG ATG GCY ACC CCH TCG ATG MTG- 3') and HEX-down (5'-  
5 CCA TCG ATG GTT ATG TKG TKG CGT TRC CGG C -3'). The amplification reaction (50 µl) contained 2 mM dNTPs, 25 pmol of each oligonucleotide, standard 1x PCR buffer, 1,5 mM MgCl<sub>2</sub>, and 1 Unit Pwo heat stable polymerase (Boehringer) per reaction. The cycler program contained 20 cycles, each  
10 consisting of 30 sec. 94°C, 60 sec. 60-64°C, and 120 sec. At 72°C. One-tenth of the PCR product was run on an agarose gel which demonstrated that a DNA fragment was amplified. Of each different template, two independent PCR reactions were performed after which the independent PCR fragments obtained  
15 were sequenced to determine the nucleotide sequence. From 11 different serotypes, the nucleotide sequence could be compared to sequences present in the genebank. Of six other serotypes, i.e. 28, 32, 36, 49, 50, and 51, the nucleotide sequence encoding fiber protein was previously unknown.

20 To demonstrate an altered immune response towards chimeric adenoviruses, we first tested 75 sera derived from human patients (25 cancer patients, 50 rheumatoid arthritis patients) for toxicity on human 911 cells. For this purpose,  
25 911 cells were seeded at a concentration of  $3 \times 10^4$  cells per well in 96-well microtiter plates. Twenty-four hours later the medium of all wells, except for wells A1-H1, A5-H5, and A9-H9, was replaced by 50 µl DMEM supplemented with 5% fetal calf serum. To wells A1, A2, B1, and B2, 50 µl patient serum  
30 1 was added. Likewise, To wells C1, C2, D1, and D2, 50 µl of patient serum 2 was added etc. Subsequently, 50 µl of wells A2-H2 were transferred to A3-H3 after which 50 µl of wells A3-H3 was transferred to A4-H4. Thus this test schedule resulted in a serum dilution of 0x, 2x, 4x, and 8x for each  
35 patient serum. Identical treatment of wells A5-H5 through A8-H8, and A9-H9 through A12-H12 results in 12 sera tested per

96-well microtiter plate. From 75 human patient sera tested in total, 25 sera with no apparent toxicity on human 911 cells were subsequently tested for the presence of antibodies capable of neutralising chimeric adenovirus infection. For this purpose, 96-well microtiter plates were filled with 50  $\mu$ l DMEM supplemented with 5% fetal calf serum except for wells A1-H1. To wells A1, A2, B1, and B2, 50  $\mu$ l patient serum 1 was added. Likewise, To wells C1, C2, D1, and D2, 50  $\mu$ l patient serum 2 was added etc. Subsequently, 50  $\mu$ l of wells A2-H2 were transferred to wells A3-A4 after which 50  $\mu$ l of A3-H3 was transferred to A4-H4 etc. until A12-H12 (dilution range: 0 - 1/ 2048). From wells A12-H12, 50  $\mu$ l was discarded. Next, 50  $\mu$ l of virus was added after which the microtiter plates were incubated for 1 hour at 37°C. Upon the addition of 50  $\mu$ l 911 cell-suspension ( $3 \times 10^4$  cells/ well) plates were incubated for 7-9 days after which neutralising capacity was scored by the absence, presence, or severity of CPE. As controls during these experiments absence of serum, absence of virus, and absence of serum and virus were taken. Based on these experiments several chimeric viruses are identified towards which little neutralising antibodies are generated by humans. Identical experiments as described above are performed with wildtype adenovirus serotypes from both human as well as animals to screen for serotypes which are less prone to neutralisation due to the host defence system.

The above described examples 1-3 encompasses the construction of recombinant adenoviral vectors, chimeric for either fiber protein or hexon protein which results in an altered infection host range or altered immune response towards adenoviral vectors. These chimeric adenoviral vectors are generated for the purpose of gene transfer and recombinant DNA vaccines. It must be stressed that in a manner analogous as described under example 1-3 chimeric adenoviral vectors are constructed for penton and can be constructed for all other adenovirus proteins including but not limited to DNA

encoding for small proteins required for adenovirus assembly and sequences required for adenovirus replication. Moreover, it must be emphasized that with this technology double, triple, quadruple, etc chimeric adenoviral vectors can be

- 5 constructed with the aim to combine parts of existing adenovirus serotypes to generate adenoviral vectors with preferred characteristics for any given target cell or target disease.

## References

- Arnberg N., Mei Y. and Wadell G., 1997. Fiber genes of adenoviruses with tropism for the eye and the genital tract. *Virology* 227: 239-244.
- 5 Bout A., 1997. Gene therapy, p. 167-182. In: D.J.A. Crommelin and R.D. Sindelar (ed.), *Pharmaceutical biotechnology*, Harwood Academic Publishers.
- 10 Bout, A. 1996. Prospects for human gene therapy. *Eur. J. drug Met. And Pharma.* 2, 175-179.
- Blaese et al., *Cancer Gene Ther.*, 2 (1995):291-297).
- 15 Brody and Crystal, *Ann. N. Y. Acad. Sci.* 716(1994):90-101.
- Chroboczek J., Ruigrok R.W.H., and Cusack S., 1995. Adenovirus fiber, p. 163-200. In: W. Doerfler and P. Bohm (ed.), *The molecular repertoire of adenoviruses*, I. Springer-Verlag, Berlin.
- 20 Defer C., Belin M., Caillet-Boudin M. and Boulanger P., 1990. Human adenovirus-host cell interactions; comparative study with members of subgroup B and C. *Journal of Virology* 64 (8): 3661-3673.
- 25 De Jong, J.C., Wermenbol, A.G., Verweij-Uijterwaal, M.W., Slaterus, K.W., Wertheim-van Dillen, P., van Doornum, G.J.J., Khoo, S.H., and Hierholzer, J.C. (1998) Adenoviruses from HIV-infected patients, including two new candidate serotypes Ad50 and Ad51 of Subgenus D and B1 respectively. In preparation.
- 30



- Eisenlohr, L.C., Gerard, W., and Hackett, C.J. (1987). Role of receptor-binding activity of the viral hemagglutinin molecule in the presentation of influenza virus antigens to helper T-cells. *J. Virol* 61, 1375-1383
- 5 Eiz B and Pring-Åkerblom P., 1997. Molecular characterization of the type-specific g-determinant located on the adenovirus fiber. *Journal of Virology* 71: 6576-6581.
- 10 Francki, R.I.B., Fauquet, C.M., Knudson, D.L. and Brown, F. (1991) Classification and nomenclature of viruses. Fifth report of the international Committee on taxonomy of viruses. *Arch. Virol. Suppl.* 2, 140-144
- 15 Gahéry-Ségard H., Farace F., Godfrin D., Gaston J., Lengagne R., Tursz T., Boulanger P. and Guillet J., 1998. Immune response to recombinant capsid proteins of adenovirus in humans: antifiber and anti-penton base antibodies have a synergistic effect on neutralizing activity. *Journal of*
- 20 *Virology* 72: 2388-2397.
- Gall J., Kass-Eisler A., Leinwand L. and Falck-Pedersen E., 1996. Adenovirus type 5 and 7 capsid chimera: fiber replacement alters receptor tropism without affecting primary
- 25 immune neutralization epitopes. *Journal of Virology* 70 (4): 2116-2123.
- Greber, U.F., Willets, M., Webster, P., and Helenius, A. (1993). Stepwise dismantling of adenovirus 2 during entry
- 30 into cells. *Cell* 75, 477-486.
- Hynes, R.O. (1992) Integrins: versatility, modulation and signalling in cell adhesion. *Cell* 69, 11-25
- 35 Herz and Gerard, *Proc. Natl. Acad. Sci. U.S.A.*, 96 (1993):2812-2816

- Hierholzer, J.C. (1992) Adenovirus in the immunocompromised host. Clin. Microbiol Rev. 5, 262-274.
- 5 Hierholzer, J.C., Wigand, R., Anderson, L.J., Adrian, T., and Gold, J.W.M. (1988) Adenoviruses from patients with AIDS: a plethora of serotypes and a description of five new serotypes of subgenus D (types 43-47). J. Infect. Dis. 158, 804-813.
- 10 Ishibashi, M. and Yasue (1983) in Adenoviruses of Animals, Chapter 12, p497-561
- Kay, R., Takei, F., and Humphries, R.K. (1990). Expression cloning of a cDNA encoding M1/69-J11d heat-stable antigens.
- 15 J. Immunol. 145 (6), 1952-1959
- Khoo, S.H., Bailey, A.S., De Jong, J.C., and Mandal, B.K. (1995). Adenovirus infections in human immunodeficiency virus-positive patients: Clinical features and molecular
- 20 epidemiology. J. Infect. Dis 172, 629-637
- Kidd, A.H., Chrboczek, J., Cusack, S., and Ruigrok, R.W. | H. (1993) Adenovirus type 40 virions contain two distinct fibers. Virology 192, 73-84.
- 25 Krasnykh V.N., Mikheeva G.V., Douglas J.T. and Curiel D.T., 1996. Generation of recombinant adenovirus vectors with modified fibers for altering viral tropism. Journal of Virology 70(10): 6839-6846.
- 30 Krasnykh V., Dmitriev I., Mikheeva G., Miller C.R., Belousova N. and Curiel D.T., 1998. Characterization of an adenovirus vector containing a heterologous peptide epitope in the HI loop of the fiber
- 35 knob. Journal of Virology 72(3): 1844-1852.

- Leopold, P.L., Ferris, B., Grinberg, I., Worgall, S., Hackett, N.R., and Crystal, R.G. (1998). Fluorescent virions: Dynamic tracking of the pathway of adenoviral vectors in living cells. *Hum. Gene Ther.* 9, 367-378.
- 5 Levrero, M., Barban, V., Manteca, S., Ballay, A., Balsamo, C., Avantaggiata, M.L., Natoli, G., Skellekens, H., Tiollais, P., and Perricaudet, M. (1991). Defective and non-defective adenovirus vectors for expression foreign genes in vitro and  
10 in vivo. *Gene* 101, 195-202.
- Matlin, K.S., Reggio, H., Helenius, A., and Simons, K. (1981). Infectious entry pathway of influenza virus in a canine kidney cell line. *J. Cell Biol.* 91, 601-613
- 15 Morgan, C., Rozenkrantz, H.S., and Mednis, B. (1969). Structure and development of viruses as observed in the electron microscope. X. Entry and uncoating of adenovirus. *J. Virol* 4, 777-796.
- 20 Richman, D.D., Hostetler, K.Y., Yazaki, P.J., and Clark, S. (1986). Fate of influenza A virion proteins after entry into subcellular fractions of LLC cells and the effect of amantadine. *Virology* 151, 200-210
- 25 Stevenson S.C., Rollence M., White B., Weaver L. and McClelland A., 1995.  
Human adenovirus serotypes 3 and 5 bind to two different cellular receptors via the fiber head domain. *Journal of*  
30 *Virology* 69(5): 2850-2857.
- Stevenson S.C., Rollence M., Marshall-Neff J. and McClelland A., 1997. Selective targeting of human cells by a chimeric adenovirus vector containing a modified fiber protein.  
35 *Journal of Virology* 71(6): 4782-4790.

- Signas, G., Akusjarvi, G., and Petterson, U. (1985). Adenovirus 3 fiberpolypeptide gene: Complications for the structure of the fiber protein. *J. Virol.* 53, 672-678.
- 5 Stouten, P.W.F., Sander, C., Ruigrok, R.W.H., and Cusack, S. (1992) New triple helical model for the shaft of the adenovirus fiber. *J. Mol. Biol.* 226, 1073-1084.
- 10 Schulick, A.H., Vassalli, G., Dunn, P.F., Dong, G., Rade, J.J., Zamarron, C. and Dichek, D.A. (1997). Established immunity precludes adenovirus-mediated gene transfer in rat carotid arteries.
- 15 Schnurr, D and Dondero, M.E. (1993) Two new candidate adenovirus serotypes *Intervirology* 36, 79-83
- Svensson, V. and Persson, R. (1984). Entry of adenovirus 2 into Hela cells. *J. Virol.* 51, 687-694.
- 20 Varga, M.J., Weibull, C., and Everitt, E. (1991). Infectious entry pathway of adenovirus type 2. *J. Virol* 65, 6061-6070.
- 25 Wickham T.J., Carrion M.E. and Kovesdi I., 1995. Targeting of adenovirus penton base to new receptors through replacement of its RGD motif with other receptor-specific peptide motifs. *Gene Therapy* 2: 750-756.
- 30 Wickham T.J., Segal, D.M., Roelvink, P.W., Carrion M.E., Lizonova, A., Lee, G-M., and Kovesdi, I. (1996). Targeted adenovirus gene transfer to endothelial and smooth muscle cells by using bispecific antibodies. *J. Virol.* 70 (10), 6831-6838
- 35 Wickham, T.J., Mathias, P., Cherish, D.A., and Nemerow, G.R. (1993) Integrins  $\alpha v \beta 3$  and  $\alpha v \beta 5$  promote adenovirus internalisation but not virus attachment. *Cell* 73, 309-319

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CLAIMS

1. A chimaeric adenovirus comprising at least a part of a fiber protein of an adenovirus serotype providing the chimaeric virus with a desired host range and at least a part of a penton or hexon protein from another less antigenic adenovirus serotype resulting in a less antigenic chimaeric adenovirus.
2. A recombinant vector derived from an adenovirus comprising at least one ITR and a packaging signal having an insertion site for a nucleic acid sequence of interest, and further having an insertion site for functionally inserting a gene encoding a penton and/or a hexon protein of a first serotype of adenovirus and having an insertion site for a gene encoding a fiber protein of a second adenovirus of a different serotype.
3. A recombinant vector according to claim 2 which is a plasmid.
4. A packaging cell for producing a chimaeric adenovirus according to claim 1, comprising in trans all elements necessary for adenovirus production not present on the adenoviral vector according to claim 2.
5. A kit of parts comprising a packaging cell according to claim 4 and a recombinant vector according to claim 2 or 3, whereby there is essentially no overlap leading to recombination resulting in the production of replication competent adenovirus between said cell and said vector.
6. A vector according to claim 2 or 3 where the insertion sites are different and preferably unique restriction sites.
7. A method for producing a chimaeric adenovirus having a desired host range and diminished antigenicity, comprising providing a vector according to claim 2, inserting into said vector at least a functional part of a penton or hexon protein derived from an adenovirus serotype having relatively low antigenicity, inserting at least a functional part of a fiber protein derived from an adenovirus serotype having the

desired host range and transfecting said vector in a packaging cell according to claim 4 and allowing for production of chimaeric viral particles.

5 8. A method according to claim 7, wherein said reduced antigenicity is a deminished capability to raise neutralizing antibodies.

9. A chimaeric adenovirus according to claim 1, wherein the hexon, penton and/or fiber proteins are chimaeric proteins originating from different adenovirus serotypes.

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ABSTRACT

The present invention provides methods and vector systems for the generation of chimeric recombinant adenoviruses. These hybrid adenoviruses contain a genome that is derived from different adenovirus serotypes. In particular, novel hybrid adenoviruses are disclosed with improved properties for gene therapy purposes. These properties include: a decreased sensitivity towards neutralising antibodies, a modified host range, a change in the titer to which adenovirus can be grown, the ability to escape trapping in the liver upon *in vivo* systemic delivery, and absence or decreased infection of antigen presenting cells (APC) of the immune system, such as macrophages or dendritic cells.

These chimeric adenoviruses thus represent improved tools for gene therapy and vaccination since they overcome the limitations observed with the currently used serotype subgroup C adenoviruses.

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